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Chemoattractant-Mediated Rap1 Activation and Localization of Cortexillin I during Cell Migration

조 선 대 학 교 대 학 원

생 물 학 과

차 인 준

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주화성 인자-매개 Rap1의 활성 및 세포이동 시 Cortexillin I의 위치

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지도교수 전택중

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조 선 대 학 교 대 학 원

생 물 학 과

차 인 준

차인준의 석사학위논문을 인준함.

위원장 조선대학교 교수 김영곤 (인)

위 원 조선대학교 교수 박현용 (인)

위 원 조선대학교 교수 전택중 (인)

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조선대학교 대학원

목 차

LIST OF FIGURES.....iii
ABBREVIATIONS iv
ABSTRACT v
국문초록 viii

Ⅰ. INTRODUCTION..... 1

Ⅱ. MATERIALS AND METHODS..... 5
Ⅱ-1. Materials and cell culture..... 5
Ⅱ-2. Strains and plasmids..... 5
Ⅱ-3. Rap1 activation pull-down assay..... 5
Ⅱ-4. *In vivo* Rap1 activation assay using RalGDS-YFP 6
Ⅱ-5. Development and chemotaxis analysis..... 7
Ⅱ-6. Quantitation analysis of GFP fusion proteins 8

Ⅲ. RESULTS 9
PART I. Chemoattractant-Mediated Rap1 Activation via GPCR/G-proteins 9
GPCRs/G proteins are required for chemoattractant-mediated Rap1 activation 9
Chemoattractant-mediated Rap1 activation is independent of cGMP signaling pathway..... 14

PART II. Localization of Cortexillin I during Cell Migration 19

Cortexillin are required for formation of cell polarity, cell shape, and multicellular development 19

Subsellular localization of cortexillin I 23

Translocation kinetics of cortexillin I in response to chemoattractant stimulation 26

Localization of GFP-ArpD in *ctxA/B* null cells 30

Localization of GFP-coronin in *ctxA/B* null cells 33

IV. DISCUSSION 35

V. REFERENCES ..... .42

VI. ACKNOWLEDGEMENTS ..... .46

LIST OF FIGURES

Fig. 1. Localization of RalGDS-YFP, a Rap1-GTP reporter, in cells lacking GPCR/G proteins..... 11
Fig. 2. Chemoattractant-mediated Rap1 activation in cells lacking GPCR/G proteins..... 13
Fig. 3. Localization of RalGDS-YFP, a Rap1-GTP reporter, in cGMP signaling mutants.. 16
Fig. 4. Chemoattractant-mediated Rap1 activation in cGMP signaling mutants... 18
Fig. 5. Chemotaxis and multicellular development of ctxA/B null cells.... 21
Fig. 6. Spatial localization of GFP-cortexillin I 25
Fig. 7. Temporal localization of GFP-cortexillin I in response to chemoattractant stimulation... 28
Fig. 8. Localization of GFP-ArpD in ctxA/B null cells..... 31
Fig. 9. Localization of GFP-coronin in ctxA/B null cells.... 34

ABBREVIATIONS

cAMP	Cyclic adenosine monophosphate
cAR	Cyclic AMP receptors
cGMP	Cyclic guanosine monophosphate
GAP	GTPase activating proteins
Gbp	cGMP-binding protein
GCA	Guanylate cyclase activator
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GPCR	G-protein coupled receptor
GTP	Guanosine triphosphate
PIP2	Phosphatidylinositos 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
RBD	Ras-binding domain
SDS	Sodium dodecyl sulfate
SGC	Soluble guanylyl cyclase
YFP	Yellow fluorescent protein

ABSTRACT

Chemoattractant-Mediated Rap1 Activation and Localization of Cortexillin I during Cell Migration

Injun Cha

Advisor: Assistant Prof.Taeck Joong Jeon, Ph.D.

Department of biology,

Graduate School of Chosun University

Chemotaxis, the directed movement of cells in response to extracellular chemoattractants, plays an important role in various biological processes, including angiogenesis, inflammation, wound healing and metastasis of cancer. Cell adhesion and cytoskeletal rearrangement is very important for proper migration. Small GTPase Rap1 is involved in the control of diverse cellular processes, including integrin-mediated cell adhesion, cadherin-based cell-cell adhesion, and cell polarity in mammalian cells, as well as cell adhesion, phagocytosis, and cell migration in *Dictyostelium*. Cortexillins are actin-bundling proteins that play a critical role in regulating cell morphology and actin cytoskeleton reorganization.

In this study, first I investigated chemoattractant-mediated Rap1

signaling pathway (PART I) and demonstrated that G-protein coupled receptors and G-proteins are essential for chemoattractant-mediated Rap1 activation in *Dictyostelium*. The rapid Rap1 activation upon cAMP chemoattractant stimulation was absent in cells lacking chemoattractant cAMP receptors cAR1/cAR3 or a subunit of the heterotrimeric G-protein complex Gα2. Loss of guanylyl cyclases GCA/SGC or a cGMP-binding protein GbpC exhibited no effect on Rap1 activation kinetics.

Next, I examined dynamic subcellular localization of cortexillin I in chemotaxing *Dictyostelium* cells (PART II). Most of the cortexillin I was enriched on the lateral sides of moving cells. Upon chemoattractant stimulation, cortexillin I was rapidly released from the cortex followed by a transient translocation to the cell cortex with a peak at ~5 sec and a subsequent decrease to basal levels, indicating that localization of cortexillin I at the cortex in chemotaxing cells is controlled by two more signaling components, one for the initial delocalization from the cortex and another for the translocation to the cortex ~5 sec after chemoattractant stimulation. Loss of cortexillins leads to reduced cell polarity and an increased number of lateral pseudopodia during chemotaxis. Cells lacking cortexillins displayed extended chemoattractant-mediated Arp2/3 complex translocation kinetics to the cortex.

These results suggest that Rap1, a key regulator for the regulation of cytoskeletal reorganization during cell movement, is activated through the G-protein coupled receptors cAR1/cAR3 and

Gα2 proteins in a way independent of the cGMP signaling pathway. Furthermore, my present study provides a new insight into the function of cortexillins during reorganization of the actin cytoskeleton and cell migration.

국문초록

주화성인자-매개 Rap1의 활성 및 세포이동 시 Cortexillin I의 위치

세포 외부의 주화성 인자에 반응하여 이동하는 주화성이동은 다양한 생물학적 과정에 중요한 역할을 한다. 예를 들어 혈관형성, 염증, 상처치유, 암 전이가 있다. 세포 부착과 세포골격 재배치는 세포의 적절한 이동에 매우 중요하다. Small GTPase Rap1은 다양한 세포의 과정을 조절하는 역할을 한다. 이러한 과정으로는 Mammalian 세포에서 integrin 매개 세포 부착과, cadherin 기반 세포 세포 부착, 세포극성 뿐만 아니라, *Dictyostelium* 에서의 세포부착, 식세포 작용, 세포이동이 있다. Cortexillin은 actin-bundling 단백질로 세포의 형태조절과 actin 세포골격 재배치에 결정적인 역할을 한다.

이 연구에서는 첫째로, 주화성 인자-매개 Rap1 신호 전달 경로를 조사하였고 (PART I), *Dictyostelium* 에서 주화성 인자-매개 Rap1활성을 위해 G-단백질 과 GPCRs가 필수적임을 입증 했다. 주화성인자 cAMP 수용체인 cAR1/cAR3 와 G-단백질 혼합체인 Gα2가 없는 세포에서 주화성인자 cAMP에 의한 신속한 Rap1 활성이 없었다. Guanylyl cyclases GCA/SGC 또는 cGMP-binding 단백질인 GbpC가 없을 때 Rap1 활성에 영향을 주지 않았다.

둘째로 주화성 이동하는 *Dictyostelium* 에서 Cortexillin I의 위치를 조사하였다 (PART II). 대부분 Cortexillin I 은 움직이는 세포의 옆 가장자리에 위치했다. Cortexillin I은 주화성 인자의 자극에 의해 세포 피질부분에서 약 5초에 최고정점을 이루며 일시적으로 증가하였다가

기저 수준으로 신속하게 감소하였다. 이 결과는 주화성 이동하는 세포에서 Cortexillin I의 위치는 두 개의 신호전달 구성요소에 의해 조절된다는 것을 시사해 준다. 하나는 세포 피질에서의 감소에, 다른 하나는 ~5초에 세포피질로의 위치이동에 작용할 것으로 생각된다. 주화성 이동하는 세포에서 Cortexillin이 없으면 세포는 극성이 감소하고 측면 위족의 수가 증가하였다. 또한 주화성인자에 의한 Arp2/3 복합체의 일시적인 세포 피질로의 위치이동이 야생종에 비해 보다 오랫동안 지속되었다.

이러한 결과는 세포 이동 시 세포골격의 재배치를 조절하는 Rap1은 G-단백질 연관 수용체 cAR1/cAR3 와 Gα2 단백질을 통해서 활성화 되며, cGMP 신호경로는 무관함을 나타내준다. 또한 이 연구는 actin 세포골격의 재배치와 세포이동 시 Cortexillin의 기능에 대한 새로운 역할에 대한 이해에 도움이 될 것으로 사료된다.

Ⅰ. Introduction

Chemotaxis, the directed movement of cells in response to extracellular chemoattractants, is an essential cellular response. Chemotaxis is involved in many diverse physiological processes, including vascular disease, osteoporosis, chronic inflammatory diseases such as rheumatoid arthritis and multiple sclerosis, cancer, and mental retardation. Misguided cell migration results in a variety of human diseases (Kortholt and van Haastert, 2008; Ridley, 2003).

Dictyostelium discoideum is a powerful model system for understanding the mechanisms of signaling pathway during eukaryotic chemotaxis (Van Haastert and Devreotes, 2004). *Dictyostelium* has several advantages in molecular and cellular experiments. The genome of *Dictyostelium*, which sequencing has been completed, contains many genes that are homologous to those of higher eukaryotes (Eichinger, 2005). As the mechanism of chemotaxis is essentially identical in all eukaryotes, *Dictyostelium* offers a genetically manageable model in the chemotaxis study (Janetopoulos and Firtel, 2008; King and Insall, 2009; Landree and Devreotes, 2004).

The first step in chemotaxis is binding of chemoattractants to cell surface G-protein coupled receptors (GPCRs). Upon ligand binding, GPCRs activate heterotrimeric G proteins and promote dissociation of the three subunits such as Gα-GTP and a Gβγ dimmer. Both of the activated G-protein subunits subsequently regulate a diverse set of downstream effectors and help convert the extracellular signal into the cell interior`s (Jin et al., 2008; Kortholt and van Haastert, 2008; Ridley, 2003; Sasaki and Firtel, 2006). The binding of chemoattractant cAMP to the surface

receptors activates several signaling cascades that cause cells to crawl toward the source of cAMP (Chisholm and Firtel, 2004; Manahan et al., 2004). In *Dictyostelium*, four cAMP receptors (cAR) have been identified (Dormann et al., 2001) Of the identified receptors, cAR1 has a high affinity for cAMP and is essential for early development and chemotaxis (Dormann et al., 2001). One G-protein β subunit and one Gγ subunit have been identified (Manahan et al., 2004). Of the 11 identified Gα subunits, Gα2, coupled to the cAR1 receptor, is the most important for cAMP-mediated chemotaxis (Kumagai et al., 1989; Rietdorf et al., 1997).

Small GTPase Rap1 is involved in the control of diverse cellular processes, including integrin-mediated cell adhesion, cadherin-based cell-cell adhesion, and cell polarity in mammalian cells as well as cell adhesion, phagocytosis, and cell migration in *Dictyostelium* (Bos, 2005; Jeon et al., 2007b; Lau and Haigis, 2009). Rap1 is the closest homologue of the small GTPase Ras and, like Ras, cycles between an inactive GDP-bound and an active GTP-bound form (Bos, 2005). Recently it has been reported that spatial and temporal regulation of Rap1 activity by Rap1 GTPase-activating proteins (GAPs), RapGAP1, RapGAPB, and RapGAP3 is required for proper cell migration, cell differentiation, and development (Jeon et al., 2007a; Jeon et al., 2009; Parkinson et al., 2009). GbpD, which was previously identified as a putative cGMP-binding protein and contains a CDC25-homology domain, is known as a Rap1-specific guanine nucleotide exchange factor (GEF) (Kortholt et al., 2006).

Several studies in Dictyostelium have revealed important roles of Rap1 in cAMP-mediated chemotaxis. Rap1 is rapidly and transiently activated upon chemoattractant stimulation. The activated Rap1 regulates cell adhesion and helps establish cell polarity by locally modulating myosin II assembly and disassembly through the Rap1/Phg2 signaling pathway (Jeon et al.,

2007a; Jeon et al., 2007b). However, the molecular mechanism underlying Rap1 activation by chemoattractant stimulation is not yet clearly understood. Here, I demonstrate that GPCRs and G proteins are essential for chemoattractant-mediated Rap1 activation (Specific Aim I). Furthermore, our data suggest that Rap1 activation upon chemoattractant stimulation is independent of cGMP signaling.

For proper migration, cells must coordinate F-actin-mediated protrusion at the leading edge and actomyosin contraction at the cell`s posterior. The differential polymerization of F-actin at the leading edge of the cell is regulated by numerous actin-modifying proteins such as the Arp2/3 complex, WAVE/SCAR, WASP, and ADF/cofilin (Firat-Karalar and Welch, 2011; Ridley, 2003; Sasaki and Firtel, 2006). The Arp2/3 complex directly nucleates actin assembly and forms new branch points. Efficient actin nucleation requires its interactions with WASP family members, which are Cdc42 and Rac effectors (Raftopoulou and Hall, 2004), and/or a pre-existing actin filament (Firat-Karalar and Welch, 2011; Raftopoulou and Hall, 2004; Rodal, 2005).

Cortexillin have been identified in Dictyostyelium as actin-bundling proteins that organize actin filaments preferentially into anti-parallel bundles and associate them into three dimensional meshworks (Faix et al., 1996). This activity is crucial for cytokinesis and cell morphology in *Dictyostelium*. Mutants lacking both cortexillin I and II display spread and flattened morphology and severely impaired cytokinesis, leading to the formation of large multinucleate cells (Faix, 2002; Faix et al., 1996; Weber and Faix, 1999). Cortexillin I is an F-actin bundling protein containing three domains (Faix, 2002; Faix et al., 1996; Weber and Faix, 1999). The N-terminal halves of cortexillin encompass two conserved actinbinding domains of the α-actinin/spectrin type (Faix, 2002; Faix et al., 1996). A

coiled-coil domain required for forming dimmers is located at the central region. The C-terminal region is essential for targeting to the cleavage furrow during cytokinesis and for actin-bundling activity. The last positively charged nonapeptide motif has a phosphatidylinositol (4,5)bisphosphate (PIP2)-binding site (Stock et al., 1999). Cortexillin I forms a complex with RacI and the IQGAPs DGAP1 and GAPA (Faix et al., 2001; Lee et al., 2010). The IQGAP/cortexillin complexes are involved in regulating cortical mechanics. It has been recently suggested that myosin II and IQGAP/cortexillin play important roles in regulating the ability of cells to restrict the F-actin assembly site and pseudopod formation at the leading edge of moving cells (Jeon et al., 2007b; Lee et al., 2010).

To further understand the function of cortexillins in the regulation of actin cytoskeleton reorganization during cell migration, I have investigated the dynamic subcellular localization of cortexillin I in moving *Dictyostelium* cells (Specific Aim II). My data suggest that the localization of cortexillin I at the lateral sides of moving cells is related to an inhibited production of lateral pseudopodia, and cortexillins are linked to the translocation of Arp2/3 complex to the cell cortex upon chemoattractant stimulation.

Ⅱ. MATERIALS AND METHODS

Ⅱ- 1.Materials and cell culture

I obtained anti-myc antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and glutathione-Sepharose beads from Amersham Biosciences (Fairfield, CT, USA). *Dictyostelium* strains were grown in HL5 axenic media or in association with *Klebsiella aerogenes* at 22°C. The knock-out strains and transformants were maintained in 10 μg/ml blasticidin or 20 μg/ml G418.

Ⅱ- 2. Strains and plasmids

Dictyostelium wild-type KAx-3 cells, *ctxA/B*, *car1/car3, gα2, gbpC*, and *gca/sgc* null strains were obtained from the DictyBase Stock Center, and myc-tagged Rap1 and RalGDS-YFP expression vectors were obtained from R. Firtel (University of California, San Diego). For expression of GFP-cortexillin I, the full coding sequence of the cortexillin I cDNA was generated by RT-PCR and cloned into the *Bgl*II-*Xho*I site of expression vector EXP-4(+) containing a GFP fragment. The expression plasmids for GFP-ArpD, GFP-coronin, and GFP-PhdA were described previously (Jeon et al., 2007a). The plasmids were transformed into KAx-3 or *ctxA*$^{-}$/*B*$^{-}$ cells.

II - 3. Rap1 activation pull-down assay

The Rap1 activation assay was performed as described previously (Jeon et al., 2007b) with slight modifications. The Rap1-GTP-binding domain (RBD) of mammalian RalGDS was expressed in *Escherichia coli* as a GST fusion protein and purified using glutathione-coupled Sepharose beads, as described previously (Franke et al., 1997). The purified GST-RBD was used for detecting activated Rap1. Aliquots (300 μl) of aggregation-competent cells were stimulated with 15 μM cAMP and then lysed by mixing with an equal volume of lysis buffer at the indicated times. The lysates were cleared by centrifugation for 10 min followed by incubation with 10 μg GST-RBD on glutathione-Sepharose beads at 4°C for 30 min. The beads were washed and resuspended in 30 μl of 2 × sample buffer, followed by SDS-PAGE and Western blot analysis with an anti-myc antibody. For the control of input amount of total Rap1 in the assay, the same volume of aggregation-competent cells was centrifuged and resuspended in 300 μl 2 × sample buffer without any cAMP stimulation and incubation with glutathione-Sepharose beads, and then subjected to the following experiments

II - 4. *In vivo* Rap1 activation assay using RalGDS-YFP

Log-phase vegetative cells expressing YFP-fusion RBD of RalGDS proteins were washed three times with Na/K phosphate buffer and shaken

at a density of 5 × 10^6 cells/ml in Na/K phosphate buffer for 7 hr to obtain cAMP-responsive for the aggregation-competent cells. A small volume of the aggregation-competent cells (~100 μl) was placed on a 30-mm Petri dish, containing ~3 ml of Na/K phosphate buffer, with a hole covered by a glass coverslip, and the cells were allowed to adhere to the plate for 10 min. The cells were uniformly stimulated with cAMP by quickly pipetting 250 μL of 150 μM cAMP into the plate containing cells (Jeon et al., 2007b). The fluorescence images of RalGDS-YFP translocation to the membrane in response to uniform chemoattractant stimulation were taken at time-lapse intervals of 1 s for 1 min using an inverted microscope (IX71; Olympus, Tokyo, Japan) with a camera (DS-Fi1; Nikon, Tokyo, Japan). Membrane and cortical localization of YFP fusion proteins was quantified as described previously. The frames were captured using NIS-elements software (Nikon) and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The intensity of cortical YFP was measured and the level of cortical YFP was calculated by dividing the intensity before stimulation (Eo) by the intensity at each time point (Et).

II - 5. Development and chemotaxis analysis

Exponentially growing cells were harvested and washed twice with 12mM Na/K phosphate buffer (pH 6.1) and plated on Na/K phosphate agar plates at a density of 4 x 10^6cells/cm^2 (Jeon et al., 2009). The developmental morphology of the cells was examined by photographing the developing cells at the time indicated in the figures.

The chemotaxis towards cAMP and changes in the subsellular localization of proteins in response to chemoattractant stimulation were examined as

described previously (Jeon et al., 2007a). The aggregation competent cells were plated on glass-bottomed microwell plates, and then a micropipette filled with 150 μM cAMP was positioned near the cells to stimulate them. The images of chemotaxing cells were taken at time-lapse intervals of 6 s for 30 min using an inverted microscope (IX71; Olympus, Japan) with a camera (DS-Fi1; Nikon, Japan).

II - 6. Quantitation analysis of GFP fusion proteins

The quantitation of membrane or cortical localization of GFP fusion proteins was performed as described previously (Cha et al., 2010). The aggregation competent cells were allowed to adhere to the plate for 10 min. The cells were uniformly stimulated with cAMP by quickly pipetting 250 μl of 150 μM cAMP into the plate containing cells. The fluorescence images were taken at time-lapse intervals of 1 s for 1min using an inverted microscope. The frames were captured using NIS-elements software (Nikon) and analyzed using ImageJ software (National Institutes of Health, USA). The intensity of cortical GFP was measured and the level of cortical GFP was calculated by dividing the intensity before stimulation (Eo) by the intensity at each time point (Et).

Ⅲ. RESULTS

PART Ⅰ. Chemoattractant-Mediated Rap1 Activation via GPCR/G proteins

GPCRs/G proteins are required for chemoattractant-mediated Rap1 activation

In *Dictyostelium*, Rap1 is rapidly and transiently activated in response to cAMP chemoattractant stimulation and plays an important role in reorganizing cell cytoskeleton and cell-substratum attachment (Jeon et al., 2007a; Jeon et al., 2007b). It has been well established that the initial response of the cells to extracellular cAMP chemoattractant stimulation is mediated by GPCRs and heterotrimeric G proteins (Chisholm and Firtel, 2004; Jin et al., 2008; Ridley, 2003). Therefore, it is postulated that GPCRs/G proteins are required for Rap1 activation in response to chemoattractant stimulation. However, the relationship between GPCRs/G proteins and chemoattractant-mediated Rap1 activation has not yet been determined. As part of my on-going work to understand the molecular mechanism underlying Rap1 activation upon chemoattractant stimulation, I determined first whether the GPCRs and the receptor-associated G proteins are required for Rap1 activation by measuring Rap1 activation kinetics in two mutant strains deficient in the cAMP receptors cAR1/cAR3 or Gα2.

In vivo Rap1 activation can be visualized using a YFP-tagged RBD of RalGDS (RalGDS-YFP), a Rap1-GTP reporter, which binds to Rap1-GTP and thus monitors Rap1-GTP levels and its location in cells (Jeon et al.,

2007a; Jeon et al., 2007b). I prepared *car1/car3* or *gα2* null cells expressing RalGDS-YFP and investigated localization and translocation of RalGDS-YFP to the cell membrane upon uniform chemoattractant stimulation. Before chemoattractant stimulation, RalGDS-YFP was mainly localized in the cytosol of the cells (Fig. 1A). Upon cAMP chemoattractant stimulation, wild-type cells displayed a rapid accumulation of RalGDS-YFP at the cell cortex with a peak at ~5 s followed by delocalization as previously reported (Jeon et al., 2007b), suggesting that Rap1 is rapidly and transiently activated at the cell cortex in response to chemoattractant stimulation. In contrast, no such rapid and transient accumulation of RalGDS-YFP at the cell cortex was observed after chemoattractant stimulation in the two mutant strains, *car1/car3* null cells and *gα2* null cells (Fig. 1A and B). Although the mutant strains exhibited an even distribution of RalGDS-YFP in the cell cytosol before chemoattractant stimulation as in wild-type cells, the level of RalGDS-YFP in the mutant cells at the cell cortex did not change upon chemoattractant stimulation, suggesting that there is no change in the Rap1-GTP level at the cell cortex in response to chemoattractant stimulation in the absence of the cAMP receptors cAR1/cAR3 or Gα2 proteins. These results were further confirmed by the following biochemical assay.

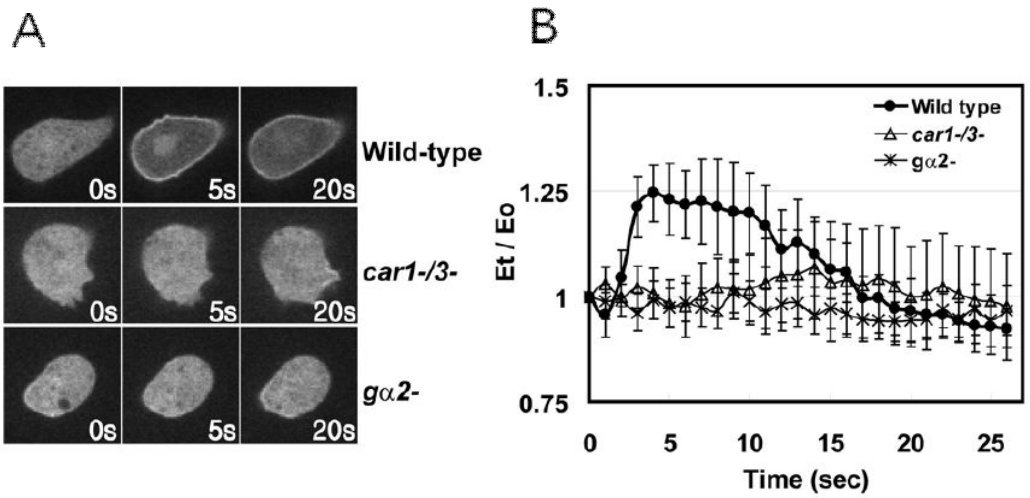

Fig. 1. Localization of RalGDS-YFP, a Rap1-GTP reporter, in cells lacking GPCR/G proteins

(A) Localization of activated Rap1 upon uniform cAMP stimulation. Translocation of RalGDS-YFP, a Rap1-GTP marker protein, to the cell cortex in response to uniform cAMP-chemoattractant stimulation in wild-type KAx-3 cells and in cells lacking GPCRs, cAR1/cAR3, or a subunit of a trimeric G protein Gα2 was imaged. The images pictured at the indicated times after stimulation were obtained from time-lapse recordings. (B) Translocation kinetics of RalGDS-YFP to the membrane were obtained from time-lapse recordings and quantified as described previously (Sasake et al., 2004). The fluorescence intensity of membrane-localized YFP fusion protein was quantified as Et/Eo. The graph represents the mean of data from several cells in videos taken from at least three separate experiments. Error bars represent ±SD.

To examine Rap1 activation kinetics in response to chemoattractant stimulation, we employed a pull-down assay using the RBD of RalGDS and anti-myc antibodies. Myc-tagged Rap1 was expressed in all strains used in these experiments and enabled us to measure Rap1-GTP levels. Consistent with the results obtained in experiments examining RalGDS-YFP localization, wild-type cells showed a rapid activation of Rap1 with a peak at 5-10 s in response to chemoattractant stimulation and then deactivation to the basal level within 40 s (Fig. 2). In contrast, the mutant strains, *car1/car3* null cells or *gα2* null cells exhibited a high basal level of Rap1-GTP before stimulation, compared to wild-type cells, and the level did not change upon chemoattractant stimulation. It is worth noting that a lack of Rap1 rapid activation in *car1/car3* null cells and *gα2* null cells upon chemoattractant stimulation might be due to reduced availability of inactive Rap1, as an elevated basal level of activated Rap1 was found in the mutant strains. In the pull-down Rap1 activation assay, the same number of cells was used to prepare total Rap1 in the T lanes and each activated Rap1 at the indicated time points (Fig. 2), but the control sample of input total Rap1 in the T lanes was diluted 10 times more than those used in the pull-down assay for detecting activated Rap1. Approximately less than 10% of the total Rap1 in *car1/3* null cells and *gα2* null cells appeared to exit as the Rap1-GTP form before stimulation, because the band intensity at the zero time point in the mutant strains was similar to that in the T lanes, implying that an elevated basal level of Rap1-GTP in the mutant strains was not a major reason for the disappearance of the rapid activation of Rap1 after stimulation. Taken together, these results suggest that the cAMP receptors cAR1/cAR3 and a component of the heterotrimeric G-protein complex Gα2 are required for rapid and transient Rap1 activation in response to cAMP chemoattractant stimulation.

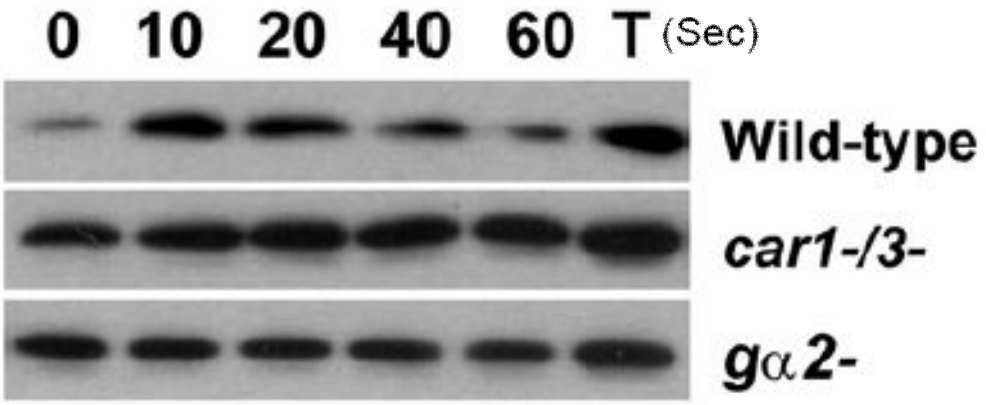

Fig. 2. Chemoattractant-mediated Rap1 activation in cells lacking GPCR/G proteins

Aggregation-competent cells were treated with 15 μM cAMP for the indicated times. The activation level of myc-tagged Rap1 was measured by pull-down assay using GST-Rap1-GTP-binding domain proteins and an anti-myc antibody. Lanes labeled 'T' show the levels of total myc-Rap1 in the same volume of each lysate.

Chemoattractant-mediated Rap1 activation is independent of cGMP signaling pathway

Myosin II filaments are formed at the cortex in the back and at the lateral sides of moving cells. Many studies have shown that cGMP is a key regulator of myosin formation (Bosgraaf and van Haastert, 2006; Ridley, 2003). Cells deficient in forming cGMP have impaired recruitment of myosin II to the cytoskeleton. cGMP is rapidly produced in response to extracellular cAMP stimulation by the enzymatic activity of two guanylyl cyclases, membrane-bound guanylyl cyclase (GCA) and soluble guanylyl cyclase (SGC) (Bosgraaf and van Haastert, 2006). GbpC is a large multidomain protein containing a RasGEF domain and two cGMP-binding domains. GbpC is only one downstream target of cGMP identified so far and is involved in cGMP-mediated chemotaxis in *Dictyostelium* (Bosgraaf and van Haastert, 2006). Recent studies have demonstrated that Rap1 is involved in the control of myosin II assembly during *Dictyostelium* chemotaxis. GbpD, a Rap1-specific GEF protein, contains cGMP-binding domains (Kortholt et al., 2006). These results raise a possibility that cGMP might be involved in the signaling pathway for Rap1 activation in response to chemoattractant stimulation. To test this possibility, I examined chemoattractant-mediated Rap1 activation kinetics in cGMP signaling mutants, *gca/sgc* null cells and *gbpC* null cells.

I first expressed RalGDS-YFP, a marker protein for Rap1-GTP, in cGMP-signaling mutant cells *gca-/sgc-* or *gbpC-*, and compared the translocation kinetics of the protein to the cell cortex to monitor the localization of the activated Rap1 in response to chemoattractant stimulation in live cells. It is noteworthy that when examining RalGDS-YFP

localization I could not compare the exact ratio of the activated Rap1 at the cell cortex to that in the cytosol of the cells, because the expression level of RalGDS-YFP in the cells varied. As in wild-type cells, RalGDS-YFP was rapidly and transiently translocated to the cell cortex in the two cGMP-signaling mutants in response to uniform chemoattractant stimulation (Fig. 3), and no apparent difference was observed between wild-type cells and the mutants in the translocation kinetics of RalGDS-YFP upon chemoattractant stimulation (Fig. 3). It suggests that Rap1 is rapidly activated in the absence of cGMP-producing proteins GCA/SGC or a cGMP-binding protein GbpC with similar activation kinetics to that in wild-type cells.

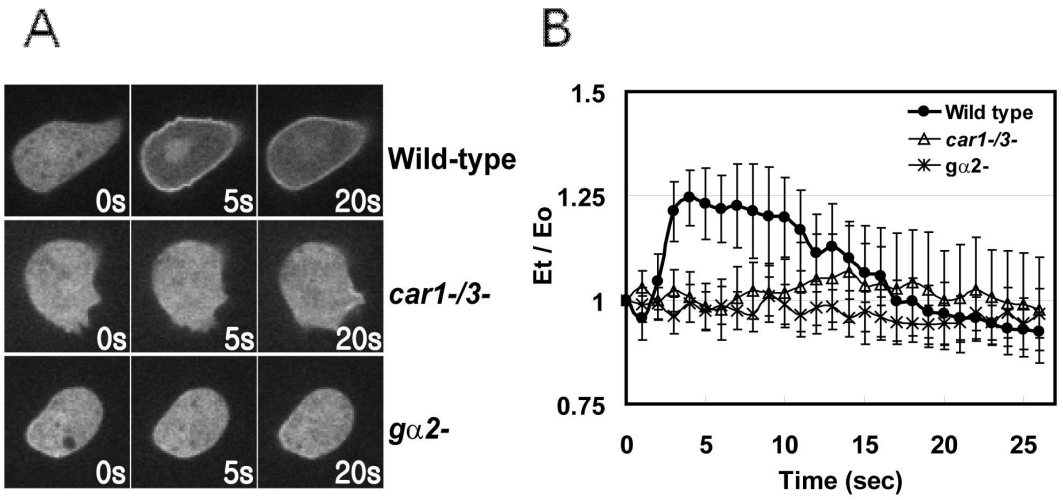

Fig. 3. Localization of RalGDS-YFP, a Rap1-GTP reporter, in cGMP signaling mutants

(A) Localization of activated Rap1 upon uniform cAMP stimulation. Translocation of RalGDS-YFP, a marker protein for Rap1-GTP, to the cell cortex in response to uniform cAMP-chemoattractant stimulation in wild-type KAx-3 cells and in *gbpC* null cells or *gca/sgc* null cells was imaged. The images were obtained from time-lapse recordings at the indicated times after stimulation. (B) Quantification and translocation kinetics of RalGDS-YFP to the membrane in cGMP signaling mutants were performed as described in the Figure 1 legend. The graphs are the means of several cells from videos from at least three separate experiments. Error bars represent ±SD.

To compare the kinetics of Rap1 activation upon cAMP stimulation in cGMP-signaling mutants with those in wild-type cells, I measured Rap1-GTP levels in the mutants and wild-type cells after chemoattractant stimulation. As suggested in the RalGDS-YFP translocation experiments, no apparent difference in the level of activated Rap1 after chemoattractant stimulation was found between mutant strains and wild-type cells (Fig. 4). All strains showed almost identical rapid activation of Rap1 in response to cAMP stimulation with a peak at ~10 sec and then the level decreased to the basal level within 40 sec (Fig. 4), indicating that the cGMP-producing proteins GCA/SGC or the cGMP-binding protein GbpC are not essential for Rap1 activation upon cAMP stimulation.

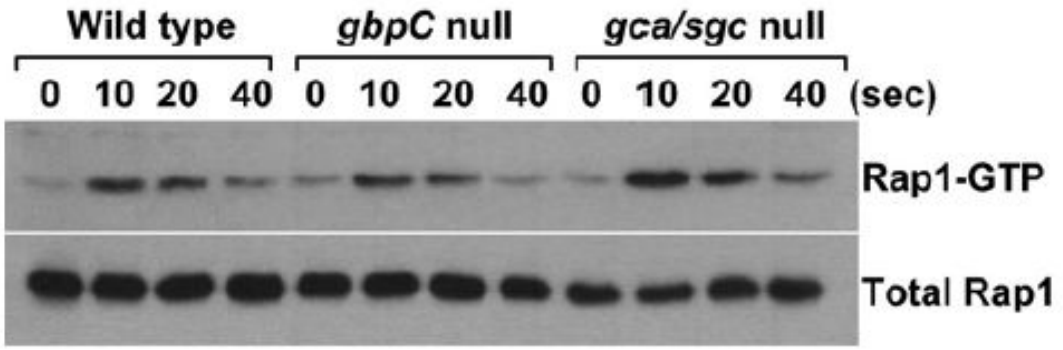

Fig. 4. Chemoattractant-mediated Rap1 activation in cGMP signaling mutants

Rap1 activation upon cAMP chemoattractant stimulation. After the cells were treated with cAMP for the indicated times, the activation level of myc-tagged Rap1 was measured by pull-down assay. Total Rap1 obtained from the samples before pulling down Rap1-GTP with GST-RBD was loaded as a loading control in the lower panel.

PART II. Localization of Cortexillin I during Cell migration

Cortexillin are required for formation of cell polarity, cell shape, and multicellular development

Cortexillin are actin-binding proteins containing three domains, whose activity is crucial for cytokinesis in *Dictyostelium* (Faix et al., 1996; Weber and Faix, 1999). Recent studies have shown that cortexillins are involved in directional cell migration by interacting with Rac and Rap1 signaling pathways (Jeon et al., 2007a; Lee et al., 2010). In this study, I examined the morphology and ability of mutant strains lacking cortexillins to polarize and chemotax up a chemoattractant gradient. Wild-type cells became elongated and polarized and chemotaxed to a micropipette emitting the chemoattractant cAMP with protrusions that were predominantly in the direction of the chemoattractant gradient (Figs. 5A and 5B). Incontrast, cells lacking both cortexillin isoforms appeared in general to be slightly larger and more flat and exhibited reduced cell polarity (increased roundness) (Figs. 5A and 5B). Cell size measurement confirmed that *ctxA/B* null cells were much larger than wild-type cells (Fig. 5C). Mean size of wild-type and *ctxA/B* null cells were 11.4 ± 2.30 and 21.7 ± 5.95 μm, respectively. In addition, *ctxA/B* null cells showed increased production of pseudopodia around the cell, including the posterior and lateral sides of cells, and a slightly slower speed of movement toward the micropipette, suggesting that cortexillins might play some roles in establishing cell polarity and inhibiting protrusion formation at the posterior and lateral sides of moving cells.

Dictyostelium cells undergo a multicellular developmental process upon starvation, eventually leading to the formation of a fruiting body within 24

h (Chisholm and Firtel, 2004). Individual cells aggregate to form a mound of 10^5 cells at approximately 10h, primarily mediated by chemotaxis to cAMP. Cells within the mound then differentiate into several cell types and form a slug-shaped structure. Culmination follows, resulting in the formation of a mature fruiting body. Because both aggregation and morphogenesis require regulated cell movement, I examined the potential involvement of cortexillins in these processes. Wild-type cells aggregated and formed mounds approximately 12h after initiating development, followed by the formation of mature fruiting bodies within 24h (Fig. 5D). In contrast, cells lacking cortexillin I and II were unable to aggregate, and development did not preceed (Fig. 5D), indicating that cortexillins are essential for multicellular development of *Dictyostelium*

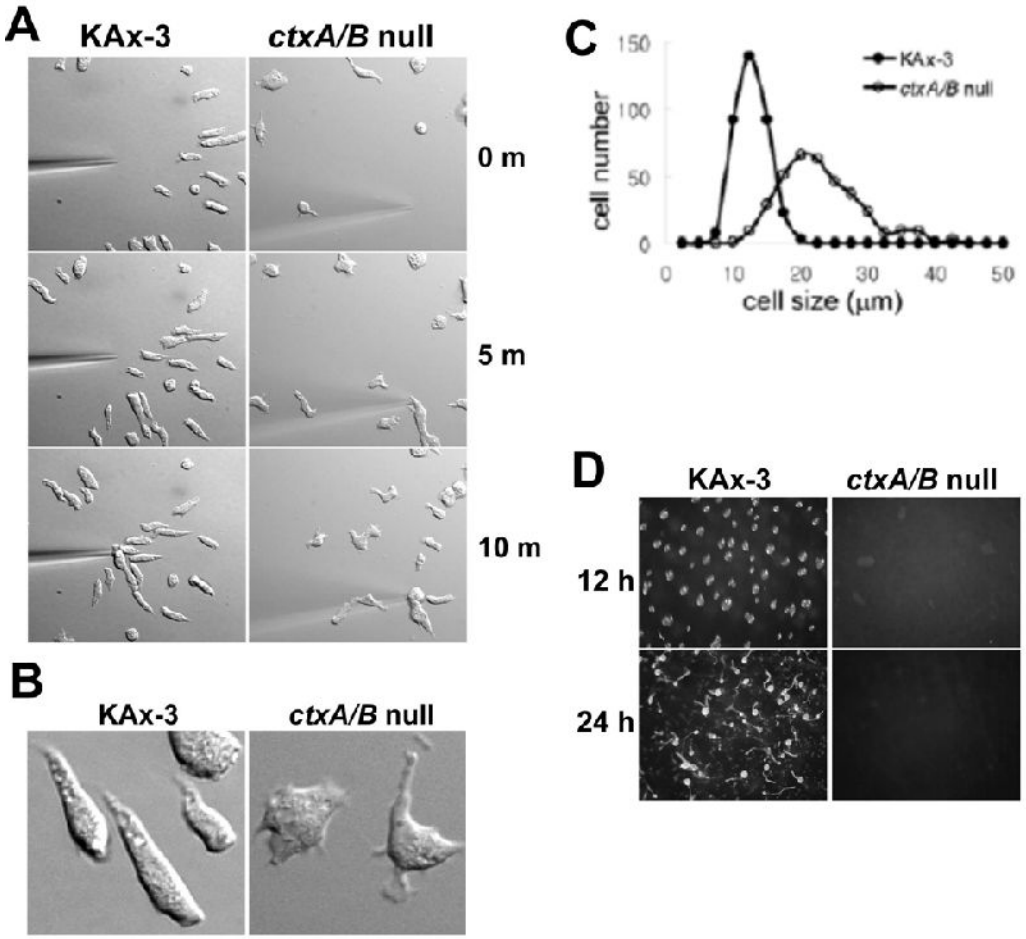
A
KAx-3
ctxA/B null
0 m
5 m
10 m
B
KAx-3
ctxA/B null
C
cell number
150
100
50
0
0
10
20
30
40
50
cell size (μm)
KAx-3
ctxA/B null
D
KAx-3
ctxA/B null
12 h
24 h

Fig. 5. Chemotaxis and multicellular development of ctxA/B null cells

(A) Chemotaxis of wild-type KAx-3 and ctxA/B null cells. Representative frames from timelapse recordings of chemotaxis are shown. The micropipette filled with 150 μM cAMP was placed on the bottom and is visible in the images. Cells chemotax toward the cAMP gradient produced by diffusion of the cAMP from the micropipette. (B) Morphology of wild-type and ctxA/B null cells. A representative image from time-lapse recordings of chemotaxing wild-type and ctxA/B null cells is shown, respectively. (C) Cell size measurement. The sizes of the vegetative wild-type and ctxA/B null cells were measured by NIS-elements software (Nikon). The histogram graphs show the distribution of the sizes of 357 cells from wild-type and ctxA/B null cells each. (D) Development of wild-type and ctxA/B null cells. Cells were developed on non-nutrient agar plates. Photographs were taken at the times indicated after plating. Development at 12 h (wild-type tip forming stage) and 24 h (wild-type fruiting body stage) is shown.

Subsellular localization of cortexillin I

I examined the localization of GFP-cortexillin I in randomly moving vegetative cells to understand dynamic roles of cortexillin in cell migration. As shown in Fig. 6A, GFP-cortexillin I localized at the plasma membrane in the resting vegetative cells, and some cells showed enrichment at the regions of membrane ruffling where F-actin accumulates (Fig. 6A). To determine whether cortexillins are localized at the F-actin assembly sites, the localization of GFP-cortexillin I was compared with that of a newly formed F-actin marker protein GFP-coronin (Gerisch et al., 1995), which associates with the Arp2/3 complex on F-actin filaments and inhibits actin nucleation (Rodal, 2005). The majority of GFP-coronin was found at protruding regions as clearly accumulated forms (Fig. 6B), which was slightly different from the relatively even distribution of GFP-cortexillin I at the cell cortex. Another clear difference in the localization of the proteins was found on the bottom of the cells. When the bottom of the cells expressing GFP-coronin were examined using a confocal microscope, small structures were found, which are known as F-actin-containing foci and may function in cell substratum adhesion (Jeon et al., 2007a). In contrast, such small structures were not found at the bottom of the cell expressing GFP-cortexillin I (Fig. 6C), indicating that cortexillin I does not localize to the actin foci at the bottom of the cell. These observations suggest that the sites where cortexillin I localizes do not match with regions of newly formed F-actin represented by GFP-coronin.

In chemotaxing cells, most coronins were enriched at the leading edge and only a small amount of protein was found in the cortex regions including the posterior and lateral sides of the cell, which is consistent with localization of the F-actin assembly (Jeon et al., 2007a)(Fig. 6E). In

contrast, GFP-cortexillin I exhibited no such enrichment at the leading edge of the moving cell as with coronin (Fig. 6D). Most of the cortexillin I was found on the plasma membrane along the lateral sides of the cell and sometimes small amounts of cortexillin I localized to the leading edge, which was a rare case, and the level of accumulated GFP-cortexillin I was much less than that of GFP-coronin (Fig. 6D). The level of cortexillin I at the posterior of the cell was significantly reduced. This unique localization of cortexillin I in moving cells suggest that cortexillin I plays some roles in the lateral sides of the cell during migration, and that binding partners of cortexillin I or binding mechanisms of cortexillin I to F-actin are likely different from those of other general actin-binding proteins such as coronin and the Arp2/3 complex, which accumulate at the leading edge of moving cells.

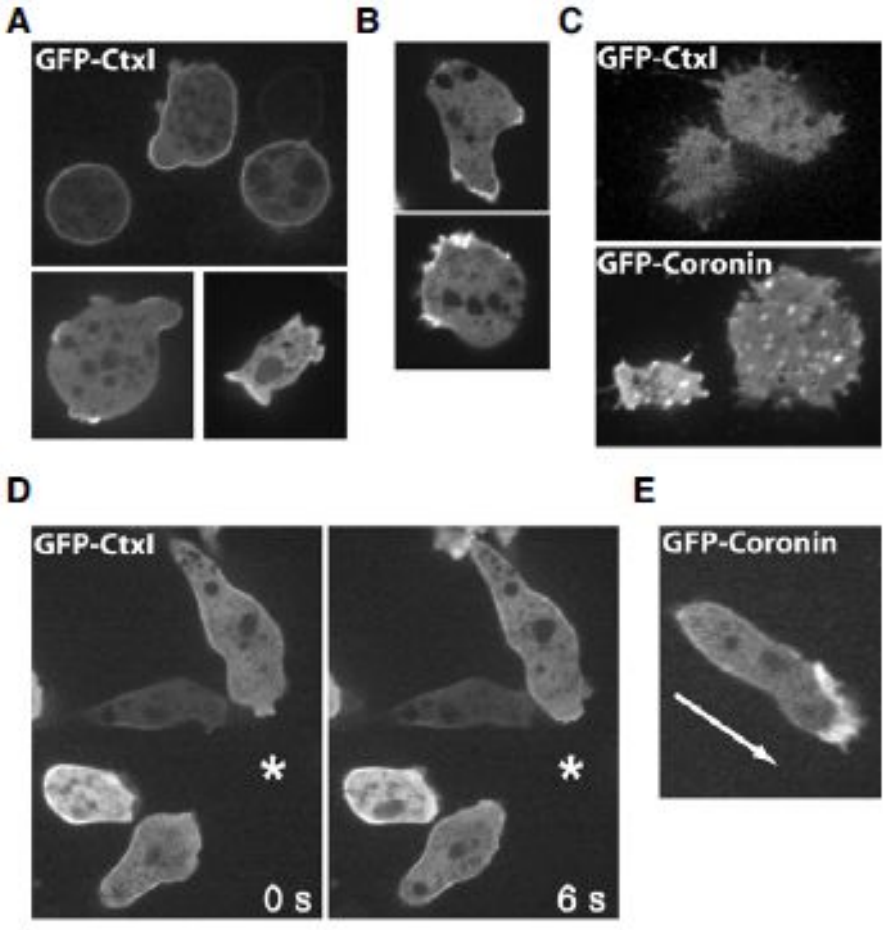

Fig. 6. Spatial localization of GFP-cortexillin I

(A) Localization of GFP-cortexillin I (GFP-CtxI) and GFP-coronin (B) in vegetative cells. Vegetative cells expressing GFP-cortexillin I or GFPcoronine were imaged. (C) Localization of GFPCtxI and GFP-coronin on the bottom of cells. Bottom sections of the cells expressing GFPctxI or GFP-coronin were imaged using a confocal microscope. (D) Spatial localization of GFPCtxI and GFP-coronin (E) in chemotaxing cells. The asterisk indicates the position of the micropipette containing cAMP. The arrow in (E) shows the direction of movement.

Translocation kinetics of cortexillin I in response to chemoattractant stimulation

I analyzed the translocation kinetics of cortexillin I to the cell cortex in response to uniform chemoattractant stimulation to understand the temporal mechanism for the dynamic localization of cortexillins to the cell cortex in chemotaxing cells. Unstimulated cells displayed quite high levels of GFP-cortexillin I at the cortex. Upon uniform chemoattractant stimulation, GFPcortexillin I at the cortex rapidly delocalized in 2-3 s shortly after stimulation and then transiently translocated to the cell cortex with a peak at ~5 s, followed by decrease of the protein amount at the cortex to the basal level within 30 s (Figs. 7A and 7B). The changes of the fluorescence intensity of GFP-cortexillin I at the cortex at 2 s and 5 s after stimulation were statistically significant, compared to that before stimulation (Fig. 7B, $p < 0.005$).

To understand temporal translocation kinetics of cortexillin I in more detail, the cell cortex translocation kinetics of cortexillin I were compared with those of other F-actin binding proteins such as ArpD, a subunit of Arp2/3 complex (Rodal, 2005), and coronin and PhdA-GFP (Funamoto et al., 2002), a pleckstrin homology domain containing PIP3 reporter (Fig. 7C). As shown in Fig. 7C, all the proteins examined in this study displayed similar rapid and transient translocation to the cell cortex with a peak at 5-10 s in response to chemoattractant stimulation. However, only GFP-cortexillin I exhibited unique initial delocalization from the cortex before transient translocation to the cortex upon stimulation (Figs. 7B and 7C), suggesting that localization of cortexillin I at the cortex is controlled by two more signaling components, one for the initial delocalization from the cortex and another for the translocation to the cortex ~5 s after

chemoattractant stimulation. Although the level of translocated cortexillin I to the cell cortex was much lower than those of other proteins and there was an initial delocalization of cortexillin I, the temporal translocation kinetics of cortexillin I upon chemoattractant stimulation was similar to that of ArpD with a maximal level of the proteins at the cortex ~5 s after stimulation, which was 1–2 s earlier than PhdA and 4–5 s earlier than coronin. The Arp2/3 complex, PhdA, and coronin are sequentially involved in regulating F-actin polymerization at the cell cortex upon chemoattractant stimulation (Firat-Karalar and Welch, 2011; Ridley, 2003). My results indicate that, within the limits of our experiments, translocation of cortexillin I to the cortex is temporally correlated with that of the Arp2/3 complex.

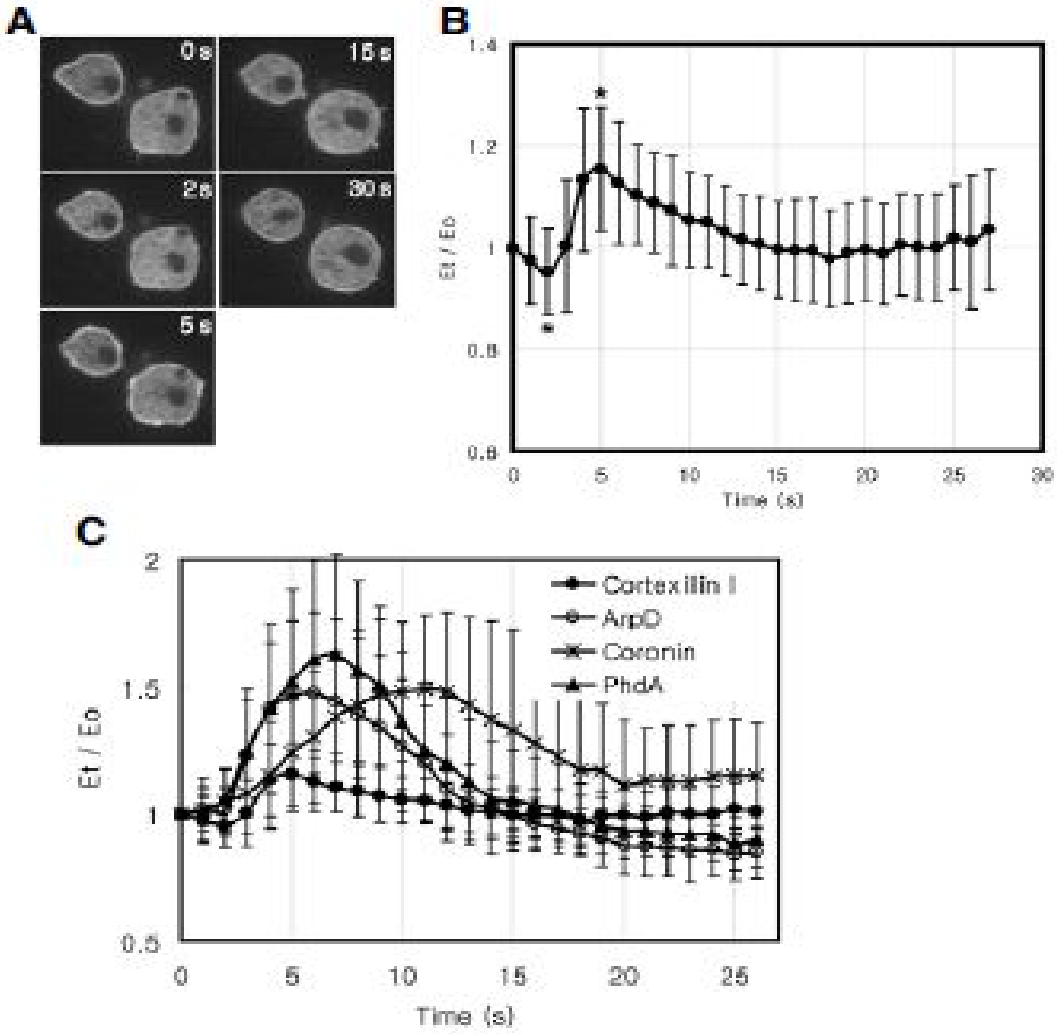
A
0 s
15 s
25
30 s
5 s
B
1.4
1.2
1
0.8
0.6
Et / Eo
0
5
10
15
20
25
30
Time (s)
C
2
1.5
1
0.5
Et / Eo
Cortexillin I
ArpD
Coronin
PhdA
0
5
10
15
20
25
Time (s)

Fig. 7. Temporal localization of GFP-cortexillin I in response to chemoattractant stimulation

(A) Translocalization of GFP-CtxI to the cell cortex in response to uniform chemoattractant stimulation. Cells were stimulated with cAMP, and the images were taken every second for 1 min. Five representative frames from time-lapse recordings are shown. (B) Translocation kinetics of GFP-CtxI. The fluorescence intensity of membrane-localized GFPCtxI was quantified. The graphs represent the means of data on several cells from videos taken from 3 separate experiments. Error bars represent S.D. (n = 52). *, statistically significant difference compared to the fluorescence intensity at 0 s (t-test, $p < 0.005$). The intensity of cortical GFP was measured and the level of cortical GFP was calculated by dividing the intensity before stimulation (Eo) by the intensity at each time point (Et). (C) Translocation kinetics of GFP-CtxI, GFP-ArpD, GFP-coronin and PhdA-GFP to the cortex were obtained from time-lapse recordings and quantified as described previously (Jeon et al., 2007a). The graphs are the means of data on several cells from at least three separate experiments

Localization of GFP-ArpD in *ctxA/B* null cells

The experiments examining translocation kinetics of the actinbinding proteins upon chemoattractant stimulation showed that the temporal translocation kinetics of cortexillin I upon chemoattractant stimulation was similar to that of ArpD, a subunit of the Arp2/3 complex. I examined the localization of GFP-ArpD in ctxA/B null cells to examine the relationship between cortexillins and the Arp2/3 complex during translocation to the cell cortex in response to chemoattractant stimulation (Fig. 8).

Both wild-type cells and ctxA-/B- cells exhibited a transient and rapid translocation of ArpD to the cell cortex in response to uniform cAMP stimulation. In chemotaxing cells, GFP-ArpD was highly accumulated at the leading edge in both wild-type and $ctxA^{-}/B^{-}$ cells. One difference was found in the translocation kinetics of ArpD to the cell cortex upon chemoattractant stimulation. Wild-type cells have a maximal level of ArpD at the cortex approximately 5-6 s after stimulation and then decrease to the basal level within 20 s, whereas $ctxA^{-}/B^{-}$ cells showed a slightly delayed and extended ArpD translocation kinetics. The maximal level of GFP-ArpD translocated to the cortex upon stimulation at approximately 8-9 s, which was 2-3 s slower than that in wild-type cells, and then the elevated level of ArpD at the cortex had a longer duration than that in wild-type cells, suggesting that cortexillins are involved in the delocalization of the Arp2/3 complex from the cortex following the transient translocation to the cortex upon chemoattractant stimulation.

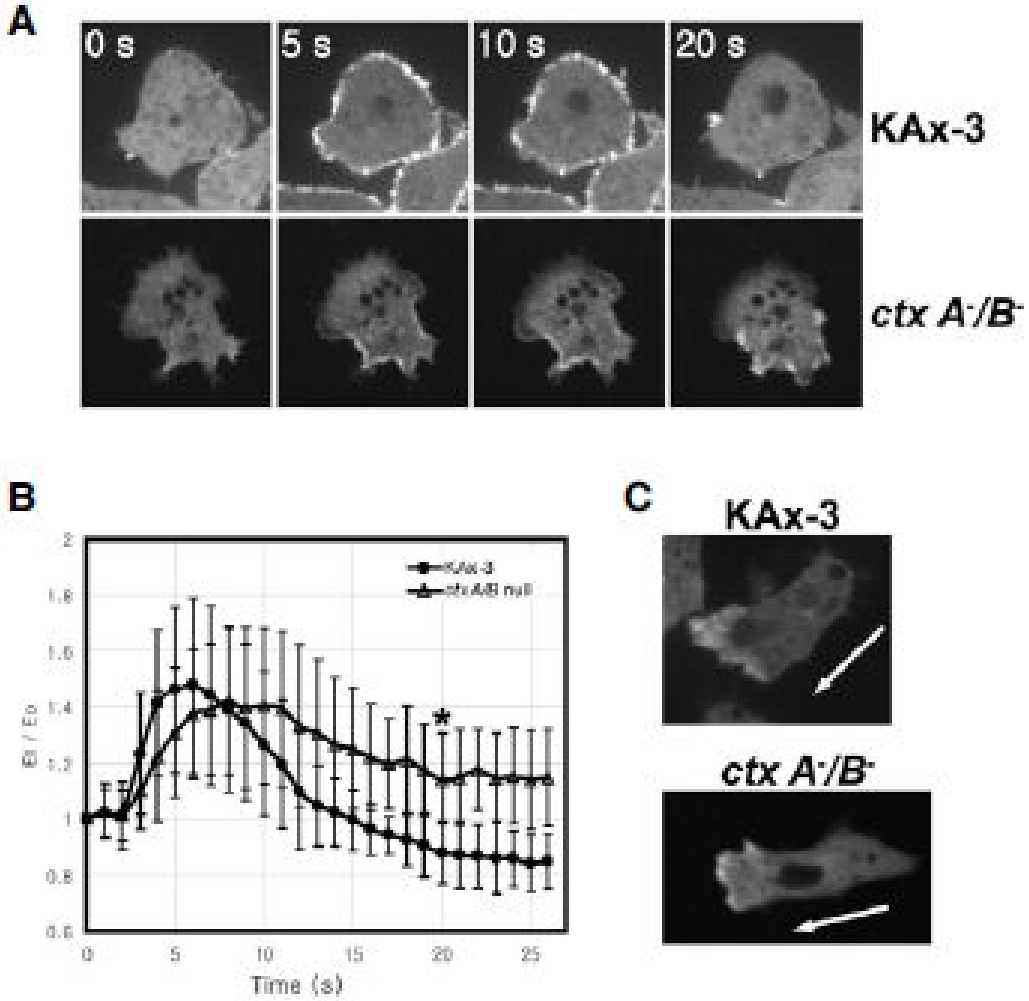
A
0 s
5 s
10 s
20 s
KAx-3
ctx A-/B-
B
KAx-3
ctxA/B null
Ei / Eo
Time (s)
C
KAx-3
ctx A-/B-

Fig. 8. Localization of GFP-ArpD in ctxA/B null cells

(A) Translocation of GFP-ArpD in wildtype KAx-3 cells and ctxA/B null cells to the cell cortex in response to uniform chemoattractant stimulation. Cells expressing GFP-ArpD were stimulated with cAMP, and the images were taken every second. Four representative frames from time-lapse recordings are shown. (B) Translocation kinetics of the GFP-ArpD in KAx-3 and ctxA/B null cells to the cell cortex. The fluorescence intensity of membrane-localized GFPArpD was quantified and graphed as described in Fig. 3. Error bars represent S.D. (n = 30). The difference at 20 s after chemoattractant stimulation between KAx-3 and ctxA/B null cells is significant (*$p < 0.001$, t-test). Et/Eo was shown in Fig7. (C) Spatial localization of GFP-ArpD in chemotaxing wild-type KAx-3 and ctxA/B null cells. The arrow indicates the direction of movement.

Localization of GFP-coronin in *ctxA/B* null cells

I examined localization of coronin in ctxA/B double null cells to determine whether localization or translocation of the other proteins involved in F-actin polymerization is also affected by the loss of cortexillins. Coronin transiently translocated to the cell cortex in response to chemoattractant stimulation in both wild-type and ctxA$^-$/B$^-$ cells (Fig. 9). The translocation kinetics of coronin in ctxA$^-$/B$^-$ cells was similar to that in wild-type cells (Figs. 9A and 9B). However, ctxA$^-$/B$^-$ cells displayed slightly lower levels of coronin translocated to the cortex upon stimulation compared to that in wild-type cells (Fig. 9B, $p > 0.1$, statistically not significant), probably resulting from the difference in coronin expression level in both cells and the flattened cell morphology of ctxA/B double null cells. In chemotaxing ctxA/B null cells, coronin localized at the leading edge as in wild-type cells. These results indicate that the localization of coronin, which binds to the Arp2/3 complex and inhibits nucleation activity of the complex (Rodal, 2005), is not affected by the absence of cortexillins.

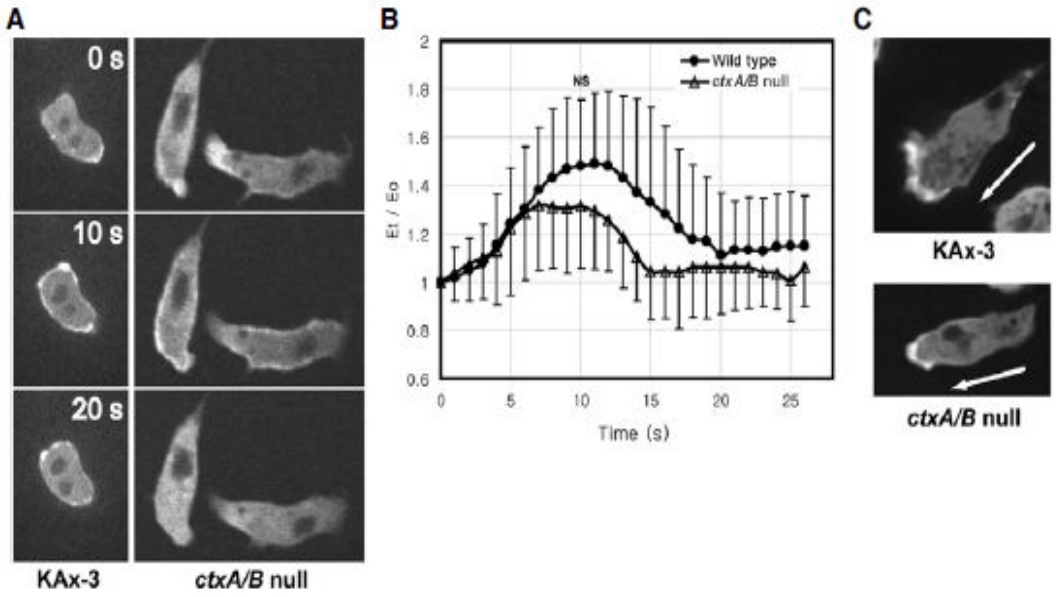

Fig. 9. Localization of GFP-coronin in ctxA/B null cells

(A) Translocation of GFP-coronin in wild-type KAx-3 cells and ctxA/B null cells to the cell cortex in response to uniform chemoattractant stimulation. Cells expressing GFP-coronin were stimulated with cAMP, and the images were taken every second. Three representative frames from time-lapse recordings are shown. (B) Translocation kinetics of the GFP-coronin in KAx-3 and ctxA/B null cells to the cell cortex. The fluorescence intensity of membrane-localized GFP-coronin was quantified and graphed as described in Fig. 3. The difference between wild-type and ctxA/B null cells is not significant (t-test, $p > 0.1$, NS, not significant). (C) Spatial localization of GFP-coronin in chemotaxing wild-type KAx-3 and ctxA/B null cells. The arrow indicates the direction of movement.

Ⅳ. DISSCUSSION

PART Ⅰ. Chemoattractant-Mediated Rap1 Activation via GPCR/G Proteins

Rap1 activation in response to cAMP

Spatial and temporal regulation of Rap1 activity is required for proper cell migration in *Dictyostelium* (Jeon et al., 2007a; Jeon et al., 2007b; Jeon et al., 2009). Misregulation of Rap1 activity in cells overexpressing constitutively active Rap1 or *rapGAP1* null cells leads to a severe chemotaxis defect and a delayed developmental phenotype during multicellular development (Jeon et al., 2007a; Jeon et al., 2007b). My present data demonstrate that a rapid and transient Rap1 activation is absent in cells lacking the cAMP receptors cAR1/cAR3 or a component of the heterotrimeric G-protein complex Gα2 in response to chemoattractant stimulation, indicating that GPCRs, cAR1/cAR3, and Gα2 proteins are essential components in the process of chemoattractant-mediated Rap1 activation. However, it is unlikely that the GPCRs, cAR1/cAR3, and Gα2 proteins are required for Rap1 activation in unstimulated cells, as there was an elevated basal level of activated Rap1 in the mutant strains before chemoattractant stimulation (Fig. 2). These results suggest that Rap1 can be initially activated without GPCRs or Gα2 and, additionally, that the cAMP receptor or G protein complex may play some role in the downregulation of Rap1 activity. In agreement with my results, several *Dictyostelium* studies have shown that other Ras proteins such as RasC and RasG play important roles in cAMP-mediated chemotaxis (Kortholt and van Haastert, 2008). Upon cAMP stimulation, both RasC and RasG are

rapidly activated and their activation is absent in mutant strains lacking cAR1/cAR3, Gβ, or Gα2 (Kae et al., 2004). Interestingly, a high basal level of activated RasC and RasG is found in the mutant strains before chemoattractant stimulation, and a negative regulatory role for the intact G-protein-coupled receptor complex in Ras activation has been suggested (Kae et al., 2004).

In mammalian cells, several positive and negative Rap1 activity regulatory mechanisms by various heterotrimeric G proteins have been suggested. As a negative control mechanism for Rap1 activation, activated α-subunits of heterotrimeric G proteins directly associate with a GAP protein for Rap1, resulting in modulation of Rap1 activity (Bos and Zwartkruis, 1999). Some GPCRs are linked to G proteins, which activate adenylyl cyclase or phospholipase C, leading to the production of intracellular second messengers such as cAMP, calcium, and diacylglycerol. These second messengers then activate GEFs for Rap1 (Bos and Zwartkruis, 1999). I do not know the exact mechanism for the activation of Rap1 via GPCRs and heterotrimeric G proteins in response to cAMP stimulation in *Dictyostelium*. In the *D. discoideum* genome, there are nine open-reading frames with a GAP domain for Rap1 and 25 genes encoding putative Ras-GEFs (Jeon et al., 2007a; Kortholt and van Haastert, 2008). Further studies are in progress to determine the interaction of Gα with Rap1GAP proteins and to characterize putative Ras-GEFs or GAP proteins for Rap1.

Activation of Rap1 in the absence of GCA/SGC or GbpC

My data suggest that even though both Rap1 and cGMP are involved in regulating myosin assembly, cGMP is not linked to the Rap1 activation signaling pathway at the initial step of Rap1 activation in response to

chemoattractant stimulation. Instead, cGMP and Rap1 may have their own distinct pathway to mediate myosin II assembly. Furthermore, my results show that loss of GbpC had no effect on chemoattractant-mediated Rap1 activation, suggesting that GbpC is unlikely to have GEF activity for Rap1. GbpC and GbpD have been identified as cGMP-binding proteins. These two proteins both contain cGMP binding domains and CDC25 homology domains. GbpC has a high affinity for cGMP and is involved in myosin II regulation (Bosgraaf and van Haastert, 2006). GbpD, homologous to the C-terminal half of GbpC (Kortholt et al., 2006), is a Rap1-specific GEF protein involved in substrate attachment and cell polarity. The gbpC null cell phenotypes, with defects in myosin II assembly, and a homologue to GbpD, a Rap1-specific GEF protein, suggest that GbpC might be an upstream regulator of Rap1 activation, but this postulate is excluded by our results. In support of our views, recent studies have shown that a RasGEF domain of GbpC has a GEF activity specific to an intramolecular Ras domain (van Egmond, 2008).

In summary, Rap1 is rapidly activated in response to chemoattractant stimulation via GPCRs cAR1/cAR3, and a heterotrimeric G-protein complex, as previously shown in other Ras proteins such as RasC and RasG. cGMP production by two guanylyl classes, GCA/sGC, or the only known cGMP-binding protein, GbpC ,are dispensable to Rap1 activation by cAMP chemoattractant stimulation.

PART II. Localization of Cortexillin I during Cell Migration

Localization of cortexillin I in moving *Dictyostelium* cells

Here, I showed that most of the cortexillin I was enriched on the lateral sides of moving cells. The localization of cortexillin I was not common to other actin-binding proteins such as coronin and the Arp2/3 complex. These proteins were accumulated at the leading edge and only small amounts of the proteins were found on the posterior or lateral sides of moving cells, which largely matched the F-actin distribution. Consistent with this result, cortexillin I was not found in the actin foci at the bottom of the cells where other actin-binding proteins accumulate. These data suggest that cortexillins might play important roles in the lateral sides of moving cells. These findings support the suggestions that cortexillins are involved in establishing cell polarity and inhibiting the production of lateral pseudopodia. Loss of cortexillins caused severe defects in morphology and chemotaxis, such as a spread and flattened morphology and abnormal production of lateral pseudopodia during cell migration, suggesting that cortexillins provide cortical tension along the lateral sides of the cells and inhibit protrusions at the cell cortex, similar to myosin II.

In agreement with my view, recent studies have proposed that myosin II and IQGAP/cortexillin are important negative regulators of leading-edge function and restrict the site of pseudopod formation to the leading edge of moving cells (Jeon et al., 2007b; Lee et al., 2010). In response to chemoattractant stimulation, cells rapidly activate a series of signaling pathways including Ras/Rap1 proteins, PI3K and TORC2, and their effectors Akt/PKB and PKBR1 at the leading edge of the cell, leading to F-actin polymerization, pseudopod formation, and directional movement up

the gradient (Charest et al., 2010; Kortholt and van Haastert, 2008). Disruption of myosin II or specific IQGAP/cortexillin complexes, which regulate cortical mechanics such as cortical tension, results in extended activation of PI3K and Akt/PKB, suggesting the negative roles of myosin II and IQGAP/cortexillin complexes in activating the signaling pathways (Lee et al., 2010). A distinct localization of cortexillin I different from other general actin-binding proteins was also found during cytokinesis. Cortexillins are much more strongly enriched in the cleavage furrow during cytokinesis rather than in the polar regions where F-actin and the potent actin-bundling protein P34 accumulate (Weber and Faix, 1999).

Translocation of cortexillin I to the cell cortex upon chemoattractant stimulation

My analysis of the translocation kinetics of cortexillin I to the cell cortex in response to uniform chemoattractant stimulation suggests that the dynamic subcellular localization of cortexillin I is controlled by two more signaling components and that the cortexillin I translocation to the cortex upon stimulation temporally correlates with the Arp2/3 complex. As shown in the localization of cortexillin I in moving cells, cortexillin I displays unique translocation kinetics to the cell cortex in response to chemoattractant stimulation, compared with those of other actin-binding proteins. Upon chemoattractant stimulation, there is a rapid release of cortexillin I from the cortex followed by a transient translocation to the cell cortex with a peak at ~5 s and a subsequent decrease to the basal level. An initial delocalization from the cell cortex upon stimulation is also found in the translocation kinetics of several proteins including myosin II, PTEN, and PakA (Funamoto et al., 2002; Jeon et al., 2007b), but the subsequent

transient translocation of cortexillin I to the cortex within 10 s is not observed with other proteins. The transient translocation kinetics of cortexillin I, except the initial delocalization of the proteins, is similar to that of the Arp2/3 complex, a nucleator of F-actin assembly. These data indicate that two more signaling components govern the localization of cortexillin I in response to chemoattractant stimulation; one for the initial delocalization from the cortex and another for the translocation to the cortex ~5 s after chemoattractant stimulation. Cortexillin I contains three domains, two actin-binding domains at the N-terminal, a coiled-coil domain at the central, and an actin-bundling activity containing domain at the C-terminal region. In addition, a PIP2-binding motif is located in the last nonapeptides of the protein (Faix et al., 1996; Stock et al., 1999; Weber and Faix, 1999). Several proteins have been identified as cortexillin binding partners. Cortexillin I interacts with RacI, IQGAPs, and GAPA forming a complex, which plays a key role in cytokinesis and cell migration (Lee et al., 2010). Furthermore, an interaction with Rap-GAP1 has been demonstrated. RapGAP1 translocates to the cell cortex in response to chemoattractant stimulation and localizes to the leading edge of the moving cell in an F-actin dependent manner (Jeon et al., 2007a). I propose that intracellular localization of cortexillin I at the cortex is determined by a balance among the actin-binding activity of the N-terminal and the membrane-binding activity of the C-terminal region of cortexillins, as well as additional interactions with other binding proteins.

Extended translocation of the Arp2/3 complex to the cell cortex in cells lacking cortexillin I and II in response to chemoattractant stimulation

My results indicate that cortexillins negatively regulate translocation of the Arp2/3 complex to the cell cortex upon chemoattractant stimulation. Loss of cortexillins resulted in an extension of the cortical localization of GFP-ArpD, a subunit of the Arp2/3 complex, upon chemoattractant stimulation. Coronin in ctxA/B null cells, an inhibitor of the Arp2/3 complex, displayed similar translocation kinetics to that in wild-type cells, although the amplitude of increased cortical fraction was smaller than that of wild-type cells. These data indicate that the effects of loss of cortexillins are limited to localization of the Arp2/3 complex, but not translocation of the inhibitor coronin, implicating that F-actin nucleation might increase in cells lacking cortexillins. Recent studies have shown a slightly increased amount of cortical Factin in ctxA/B null cells than that in wild-type cells (Lee et al., 2010).

The negative effect of cortexillins on the translocation of the Arp2/3 complex to the cortex is consistent with the findings of studies of propagating F-actin waves at the bottom of the cell (Schroth-Diez et al., 2009). Actin waves are formed at the substrate- attached surface in migrating *Dictyostelium* cells, particularly on strongly adhesive substrates. Actin waves separate two F-actin binding proteins, the Arp2/3 complex and cortexillins. The Arp2/3 complex is exclusively localized in the internal region of the F-actin waves, whereas cortexillins are localized at the external regions of the waves (Schroth-Diez et al., 2009). Taken together, my results suggest that cortexillins might play a role in restricting the Arp2/3 complex in the internal region of the F-actin waves.

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가장 먼저 지난 27년 동안 집에서 잘 될 수 있도록 기도하시고 공부에 더욱 집중할 수 있도록 노력해 주신 아버님, 어머님께 감사 드립니다. 그리고 투덜대지만 하나뿐인 누나에게도 고맙다고 전하고 싶습니다. 더욱 나아진 모습으로 어린 학생이 아닌 사회인으로 더욱 거듭날 수 있도록 노력하겠습니다.

제가 2년간 석사 학위를 잘 마칠 수 있도록 불철주야 이끌어 주신 전택중교수님께 진심으로 감사 드리며, 논문 심사를 해주신 김영곤 교수님, 박현용 교수님께도 감사 드립니다. 또한 제가 열심히 공부하도록 지도 하여주신 최영복 교수님, 조광원 교수님 그리고 이준식 교수님께 진심으로 감사 드립니다.

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대학교생활 내내 옆에서 든든히 버팀목이 되어준 나의 동기들 고맙다. 인목이, 성민이, 준이, 효진이, 호석이, 걸량이, 유중이, 경원이, 재창이, 모두 고맙다는 말을 전하고 싶습니다. 또한 항상 챙겨주고 힘들 때 위로해 주는 유진아 고마워.

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학 과	생물학과	학 번	20107042	과 정	석사
성 명	한글: 차인준 한문: 車仁俊 영문: Injun Cha				
주 소	광주광역시 서구 쌍촌동 1254-2 번지				
연락처	e-mail : boradolly@naver.com				
논문제목	한글: 주화성인자-매개 Rap1의 활성 및 세포이동 시 Cortexillin I의 위치				
	영문: Chemoattractant-Mediated Rap1 Activation and Localization of Cortexillin I during Cell Migration				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건 아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB 구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함.
2. 위의 목적을 위하여 필요한 범위 내에서의 편집과 형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5 년으로 하고, 기간종료 3 개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 출판을 허락을 하였을 경우에는 1 개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물 이용의 허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음.
7. 소속 대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의 (0) 반대 ()

2012 년 2 월 24 일

저작자 : 차인준 (인)

조선대학교 총장 귀하